VENABLE, BAETJER, HOWARD & CIVILETTI, LLP
Including professional corporations

1201 New York Avenue, N.W., Suite 1000
Washington, D.C. 20005-3917
(202) 962-4800, Fax (202) 962-8300
www.venable.com

OFFICES IN
WASHINGTON, D.C.
MARYLAND
VIRGINIA

VENABLE
ATTORNEYS AT LAW

March 15, 2001

Assistant Commissioner for Patents
Washington, D.C. 20231

Attention: Box PCT - DESIGNATED/ELECTED OFFICE (DO/EO/US)

FORM PTO-1390 (REV 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 31671-169944
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO (If known, see 37 CFR 1.5) Not Yet Assigned
INTERNATIONAL APPLICATION NO. PCT/JP99/05069	INTERNATIONAL FILING DATE 17 September 1999	PRIORITY DATE CLAIMED: 18 September 1998
TITLE OF INVENTION - see attached pages -		
APPLICANT(S) FOR DO/EO/US - see attached pages -		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

- See attached pages for additional data –

RK/rgf

DC2DOCS1\271469

VENABLE, BAETJER, HOWARD & CIVILETTI, LLP
Including professional corporations

1201 New York Avenue, N.W., Suite 1000
Washington, D.C. 20005-3917
(202) 962-4800, Fax (202) 962-8300
www.venable.com

OFFICES IN
WASHINGTON, D.C.
MARYLAND
VIRGINIA

VENABLE
ATTORNEYS AT LAW

March 15, 2001

Assistant Commissioner for Patents
Washington, D.C. 20231

Attorney Docket: 31671-169944

Attention: PCT-DO/US

Re: International Application PCT/JP99/05069 filed September 17, 1999
Priority Claimed: Japanese Appl. No. 10/265089 filed September 18, 1998

Inventor(s): Yoshimi HOMMA et al.

Title: METHOD FOR THE DIAGNOSIS OF CELL PROLIFERATIVE DISEASE

Sir:

Submitted herewith, as the first submission, are the following for the purposes of entering the national stage for the USA under 35 U.S.C. 371**(c), immediate national examination under 35 U.S.C. 371(f) being requested**.

X International Application PCT/JP99/05069 published as WO 00/17339.

X Translation of International Application.

X Two Sheets of Formal Drawings (Figs. 1 and 2)

X Preliminary Amendment.

X Inventor Declaration.

X Information Disclosure Statement and PTO-Form 1449, International Search Report and cited references.

X Assignment, coversheet and recordation fee.

VENABLE
ATTORNEYS AT LAW

Assistant Commissioner for Patents
Washington, D.C. 20231

Fees: (see formula below) Check Enclosed

Basic National Fee $840.00/420.00.....	$860.00
Additional Claim Fee	$162.00
Assignment Recordation Fee.....	$ 40.00

TOTAL FEES FOR THE ABOVE APPLICATION... $1062.00

In the event there is attached hereto no check, or a check for an insufficient amount, please charge the fee to our Account No. 22-0261 and notify us accordingly.

Please use the following address for corresponding with all counsel of record:

VENABLE
P.O. Box 34385
Washington, D.C. 20043-9998

Respectfully submitted,

Robert Kinberg
Registration No. 26,924
Telephone: (202) 962-4800
Telefax: (202) 962-8300

RK/rgf

DC2DOCS1\271469

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE 15 MAR 2001

In Re PATENT APPLICATION of

Applicant	:	Yoshimi HOMMA et al.	
Int'l Appl. No.	:	PCT/JP99/05069	
Int'l Filing Date	:	September 17, 1999	
For	:	METHOD FOR THE DIAGNOSIS OF CELL PROLIFERATIVE DISEASE	**PRELIMINARY AMENDMENT**
Attorney Docket	:	31671-169944	**March 15, 2001**

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to calculation of the fees, please amend the claims attached to the specification as follows:

9. A DNA primer having base sequence represented by Seq. ID No. 1 or 2 used for any diagnosis in claim 1.

13. A DNA primer having base sequence represented by Seq. ID No. 5 or 6 used for any diagnosis in claim 1.

IN THE CLAIMS:

Please add the following new claims:

17. A DNA primer having base sequence represented by Seq. ID No. 1 or 2 used for any diagnosis in claim 2.

18. A DNA primer having base sequence represented by Seq. ID No. 1 or 2 used for any diagnosis in claim 3.

19. A DNA primer having base sequence represented by Seq. ID No. 1 or 2 used for any diagnosis in claim 4.

20. A DNA primer having base sequence represented by Seq. ID No. 1 or 2 used for any diagnosis in claim 5.

21. A DNA primer having base sequence represented by Seq. ID No. 1 or 2 used for any diagnosis in claim 6.

22. A DNA primer having base sequence represented by Seq. ID No. 1 or 2 used for any diagnosis in claim 7.

23. A DNA primer having base sequence represented by Seq. ID No. 1 or 2 used for any diagnosis in claim 8.

24. A DNA primer having base sequence represented by Seq. ID No. 5 or 6 used for any diagnosis in claim 2.

25. A DNA primer having base sequence represented by Seq. ID No. 5 or 6 used for any diagnosis in claim 3.

26. A DNA primer having base sequence represented by Seq. ID No. 5 or 6 used for any diagnosis in claim 4.

27. A DNA primer having base sequence represented by Seq. ID No. 5 or 6 used for any diagnosis in claim 10.

28. A DNA primer having base sequence represented by Seq. ID No. 5 or 6 used for any diagnosis in claim 11.

29. A DNA primer having base sequence represented by Seq. ID No. 5 or 6 used for any diagnosis in claim 12.

REMARKS

This Preliminary Amendment is made to eliminate multiple claim dependency. Examination on the merits of the application is requested. A marked up version showing the changes made to the claims is attached.

Respectfully submitted,

Robert Kinberg
Registration No. 26,924
VENABLE
Post Office Box 34385
Washington, DC 20043-9998
Telephone: (202) 962-4800
Telefax: (202) 962-8300

RK/rgf
DC2DOCS1\271487

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Patent Claims

9. A DNA primer having base sequence represented by Seq. ID No. 1 or 2 used for any ~~one of~~ diagnosis ~~described~~ in claim~~s~~ 1 ~~to 8.~~

13. A DNA primer having base sequence represented by Seq. ID No. 5 or 6 used for any ~~one of~~ diagnosis in claim~~s~~ 1 ~~to 4 and 10 to 12~~.

Specification

Diagnostic method for detecting cell-proliferating diseases

Technical Field

This invention relates to a diagnostic method for detecting cell-proliferating diseases characterized by analyzing the methylation level of cytosine residues in the region involved in the expression of cytokine receptor gene.

Background Art

DNA of eukaryotes happens to be methylated at cytosine residue of 5th position [Cell, 70, 5-8 (1992), Blood, 93, 4059-4070 (1999)]. It is known that the state of methylation of genome DNA of mammalians varies with differentiation or canceration of cells. Methylation reaction is catalyzed by enzymes, DNA(cytosine-5)methyltransferase (EC 2.1.1.37) [BioEssays, 17, 139-145 (1995)]. The enzyme methylates cytosine residue of dinucleotide sequence CpG or trinucleotide sequence CpNpG [N can be anyone of A (adenine), C (cytosine), G (guanine) and T (thymine)]. Furthermore, recognizing specifically the state where only one strand of double strand is methylated, the enzyme methylates cytosine residue in the complementary chain. Recently the existence of an enzyme catalyzing de-methylation of DNA was suggested [Proc. Natl. Acad. Sci. USA, 96, 5894-5896 (1999)]. It is known that the state of methylation of DNA happens to be transmitted to progeny through reproduction (meiosis) by a mode of inheritance called imprinting [Trend in Genet. (TIG), 13, 323-329 (1997)].

Non-coding regions of some genes have a part called CpG island with an abundance of CpG sequence. The state of methylation of cytosine residue in CpG island affects the transcription/expression of the gene. Namely the less methylation results in the accelerated transcription of the gene and the more methylation results in the suppressed transcription [Trends in Genet., 13, 444-449 (1997)]. CpG island affecting gene expression frequently resides in the promoter region of the gene but a case was reported where it resided in the intron [Nature, 389, 745-749 (1997)]. As for the mechanism that DNA methylation in CpG island results in suppressed gene expression, the followings are known. Methylated CpG island is combined with a protein called MeCP2(methyl CpG binding repressor 2) and activates de-acetylation enzyme of histone. As a result neighboring chromatin structure changes into condensed form, which prohibits RNA polymerase or transcription factor from entering into promoter region, and, as a result, the transcription/expression is suppressed [J. Biochem., 125, 217-222

(1999)].

Among cell-proliferating diseases it is known that DNA methylation is involved in cancer development [Adv. Cancer Res., 72, 141-196 (1998)]. However most of the reports advocate that CpG island of a cancer suppression gene is methylated, the expression is lowered and, as a result, cells become cancerous. There has been no report that methylation of cytokine receptor gene is involved. It was also not known with cell-proliferating diseases other than cancers that methylation pattern of genome DNA is changed.

Methods to analyze the state of methylation of genome DNA are known, for example, a method using methylation sensitive restriction enzymes, a method using chemical modification by hydrazine, permanganic acids or sodium bisulfite, an immunological method using antibodies specific to methylated DNA [Nucleic Acids Res., 26, 2255-2264 (1998)], and affinity column chromatography method using MBD (methyl-CpG binding domain) like MeCP2 and DGGE (denaturing gradient gel electrophoresis) method [Proc. Natl. Acad. Sci. USA, 96, 2913-2918 (1999)]. Among them the method using sodium bisulfite has been widely used [Proc. Natl. Acad. Sci. USA, 89, 1827-1831 (1992), Nucl. Acids Res., 22, 2990-2997 (1994)].

The method is based on the following principle.

When single strand DNA produced by alkali denaturation is treated by sodium bisulfite, cytosine residues are changed into uracil residues by deamination, while methylated cytosine residues remain intact. Then polymerase chain reaction (hereinafter "PCR") is performed using thus treated DNA as template. The primer therefor is designed to correspond to the base sequence wherein cytosines in base sequence to be amplified are replaced by thymines. When amplification is performed using such primers, methylated cytosine residues in the original genome DNA are amplified as cytosines, while unmethylated cytosine residues are amplified as thymine residues. Thus methylation in the original genome DNA is detected by determining the base sequence of the PCR product.

Psoriasis is a chronic inflammatory skin disease and a cell-proliferating disease accompanying abnormal proliferation of epidermal cells. More than 2% of caucasian population contract it [The Lancet, 350, 349-353 (1997), The Lancet, 338, 227-230 (1991), The Lancet, 338, 231-234 (1991)]. They get it after becoming adult in most cases. The cause of psoriasis is yet to be solved. Although families with high incidence of psoriasis are known and there are reports suggesting the involvement of genetic factor, the cause itself is not yet known [Nature Getetics, 14, 231-233 (1996), Science, 264, 1141-1145 (1994), Arch. Dermatol., 130, 216-224 (1994)]. On the other hand there is a report that

the expression of epidermal growth factor receptor (EGF-R) at keratinocyte is accelerated at the affected part [J. Dermatol. Sci., 16, 120-128 (1998)]. While the expression of EGF-R in keratinocyte is normally induced by stimulation with interleukin-6 (IL-6), EGF-R was expressed at keratinocyte of psoriasis irrrespective of existence or absence of IL-6 stimulation.

The promoter region of EGF-R gene contains an abundance of CpG sequence and it was shown that transcription factors bind to the region [J. Biol. Chem., 266, 1746-1753 (1991), J. Biol. Chem., 263, 5693-5699 (1988)].

The sequence of the promoter region of human EGF-R gene is disclosed in accession number M38425 of GenBank database. The CpG sequence is especially abundant and the binding sequences of transcription factor are scattered in the region about 500 bases upstream from the translation initiation point (1114th) and the region about 800 bases downstream from the initiation point of the 1st intron in the base sequence [J. Biol. Chem., 266, 1746-1753 (1991), J. Biol. Chem., 263, 5693-5699 (1988)].

Conventional diagnosis of psoriasis has been mostly performed by long time observation by dermatologists according to the diagnostic standard (PASI: psoriasis area and severity index) described in Dermatologica, 157, 238-244 (1978), J. Dermatol. Sci., 16, 165-169 (1998) and the like. Such diagnosis needs experienced dermatologists, and long time and much work for observing tissue lesions and symptoms. Therefore a speedy, reliable and reproducible diagnostic method has been desired.

Chronic rheumatoid arthritis is one of systemic autoimmune diseases and a cell-proliferating disease accompanying abnormal proliferation and inflammation of arthrosynovial cells [Nippon Rinsho, 57, 333-338 (1999)]. The cause of chronic rheumatoid arthritis is yet to be solved. Although families with high incidence of chronic rheumatoid arthritis are known and the correlation with genotype of human histocompatibility antigen gene HLA-DR4 has been suggested, the cause itself is not yet known. There is a report that the activity of epidermal growth factor-like receptor 2 (erbB2/HER2/neu) is increased at the affected part [Seminars in Arthritis & Rheumatism, 21, 317-329 (1992)].

The sequence of the promoter region of human epidermal growth factor-like receptor 2 (erbB2/HER2/neu) gene is disclosed in accession number Z13970 of GenBank database. The region contains an abundance of CpG sequence and transcription factor Sp1 is suggested to bind to the region [Mol. Cell. Biol., 7, 2597-2601 (1987), Proc. Natl. Acad. Sci. USA, 84, 4374-4378 (1987), J. Biol. Chem., 265, 4389-4393 (1990), Gene 136, 361-364 (1993), Cancer Res., 54, 4193-4199 (1994)].

Conventional diagnosis of chronic rheumatoid arthritis has been performed by long

time observation by specialized medical doctors according to the diagnosis standard described in [Arthritis Reum., 31, 315-324 (1988)] and the like. Such diagnosis needs experienced medical doctors, and long time and much work for observing tissue legions and symptoms. Therefore a speedy, reliable and reproducible diagnostic method has been desired.

Disclosure of the Invention

The invention is to provide a speedy, reliable and reproducible method for diagnosing cell-proliferating diseases.

The invention relates to below mentioned (1) – (16).

(1) A diagnostic method for detecting cell-proliferating diseases characterized by determining the methylation level of cytosine residues at the specific region of genome DNA involved in the expression of cytokine receptor gene.

(2) A diagnostic method described in (1) wherein the cytokine receptor gene is the gene of a receptor selected from tyrosine kinase receptor family, serine-threonine kinase receptor family, interleukin receptor family, interferon receptor family, immunoglobulin receptor family, apoptotic receptor family and seven transmembrane receptor family.

(3) A diagnostic method described in (2) wherein the tyrosine kinase receptor gene is the gene of a receptor selected from epidermal growth factor receptor, epidermal growth factor-like receptor 2 (erbB2/HER2/neu), platelet derived growth factor receptor and vascular endothelial cell growth factor receptor.

(4) A diagnostic method described in (1) wherein the cell-proliferating disease is a cell-proliferating disease selected from psoriasis, chronic rheumatoid arthritis, arteriosclerosis, restenosis, diabetic retinopathy, retinopathy of prematurity and solid tumor.

(5) A diagnostic method described in (1) wherein the specific region is a region in CpG island of promoter or intron.

(6) A diagnostic method described in (1) wherein the specific region is a region involved in the expression of epidermal growth factor receptor gene and a region represented by the nucleotide sequence from 381st position to 962nd position in the nucleotide sequence as described in Seq. ID No. 4.

(7) A diagnostic method described in (6) characterized by determining the methylation level of 668th, 671st, 687th and 697th cytosine residues in the nucleotide sequence as described in Seq. ID No. 4.

(8) A diagnostic method described in (7) characterized by analyzing the methylation level of 668th cytosine residue in the nucleotide sequence as described in Seq. ID No. 4.

(9) A DNA primer having nucleotide sequence represented by Seq. ID No. 1 or 2 used for diagnostic method described in any one of (1) to (8).

(10) A diagnostic method described in (1) wherein the specific region is the region involved in the expression of epidermal growth factor-like receptor 2 (erbB2/HER2/neu) and represented by nucleotide sequence of Seq. ID No. 8.

(11) A diagnostic method described in (10) characterized by determining the methylation level of 268th, 276th and 288th cytosine residues in the nucleotide sequence as described in Seq. ID No. 8.

(12) A diagnostic method described in (11) characterized by analyzing the methylation level of 268th cytosine residue in the nucleotide sequence as described in Seq. ID No. 8.

(13) A DNA primer having nucleotide sequence represented by Seq. ID No. 5 or 6 used for any one of diagnostic methods described in (1) to (4) and (10) to (12).

(14) A DNA having nucleotide sequence represented by Seq. ID No. 1, 2, 5 or 6.

(15) A method of detecting the methylation level of cytosine residue(s) in the specific region of DNA involved in the expression of cytokine receptor gene.

(16) A method described in (15) wherein the method of detecting the methylation level is a method using methylation sensitive restriction enzyme, a method using chemical modification by hydrazine, permanganic acids or sodium bisulfite, an immunological method using antibodies specific to methylated DNA, affinity column chromatography method or DGGE (denaturing gradient gel electrophoresis) method.

This invention relates to a diagnostic method for detecting cell-proliferating diseases characterized by determining the methylation level of cytosine residues at the specific region of genome DNA involved in the expression of cytokine receptor gene.

Cytokine is a general term for protein cell-cell signal transduction molecules, which regulate proliferation or differentiation of animal cells.

Examples of cytokine receptors include tyrosine kinase receptor family, serine-threonine kinase receptor family, interleukin receptor family, interferon receptor family, immunoglobulin receptor family, apoptotic receptor family and seven transmembrane receptor family.

Examples of tyrosine kinase receptor family include epidermal growth factor-receptor (hereinafter "EGF-R") [J. Biol. Chem., 266, 1746-1753 (1991), J. Biol. Chem., 263, 5693-5699 (1988)], epidermal cell growth factor-like receptor 2 (erbB2/HER2/neu) [Biochim. Biophys. Acta, 1377, M25-M37 (1998), Biochim. Biohpys. Acta, 1198, 165-184 (1994), Molec. Cell. Biol., 7, 2597-2601 (1987)], platelet derived growth factor-receptor (hereinafter "PDGF-R") [J. Biol. Chem., 269, 32023-32026 (1994), Oncogene, 10, 1667-1672 (1995)], vascular endothelial growth factor-receptor (hereinafter "VEGF-R") [J.

Biol. Chem., 270, 27948-27953 (1995)].

Examples of the region involved in the expression of the above-mentioned receptor genes include the region from -152nd nucleotide to -733rd nucleotide upstream from translation initiation point of EGF-R gene, the region from -1st to -647th nucleotide upstream from translation initiation point of erbB2/HER2/neu gene [Mol. Cell. Biol., 7, 2597-2601 (1987)], the region from -1st to -2060th nucleotide upstream from transcription initiation point of PDGF-R gene [Oncogene, 10, 1667-1672 (1995)], the region from -720th to +548th nucleotide around transcription initiation point of VEGF-R gene [J. Biol. Chem., 270, 27948-27953 (1995)] and the like.

Cell-proliferating diseases are diseases like psoriasis characterized by keratin hypertrophy accompanying proliferation of epidermal cells and abnormal keratinization, chronic rheumatoid arthritis characterized by proliferation of synovial cell and villus hypertrophy, arteriosclerosis and restenosis characterized by proliferation of arterial smooth muscle cells and vascular media hypertrophy, diabetic retinopathy, retinopathy of prematurity and solid tumor characterized by proliferation of vascular endothelial cells and vascularization, and the like.

The diagnostic method is described referring to psoriasis as an example in the following.

Genome DNAs of a person to be diagnosed are collected from samples such as blood, saliva, sperm, skin tissue, tissue used for biopsy and the like respectively.

As methods for collecting genome DNAs from samples, a method described in Proc. Natl. Acad. Sci. USA, 74, 1245-1249 (1977), a method using ReadyAmp™ Genomic DNA Purification System (Promega) and the like are used.

As methods for determining the methylation level of genome DNAs obtained in the above, a method using methylation sensitive restriction enzyme described in Nucleic Acids Res., 26, 2255-2264 (1998), a method using chemical modification by hydrazine, permanganic acids or sodium bisulfite, an immunological method using antibodies specific to methylated DNA, affinity column chromatography method using MBD (methyl-CpG binding domain) such as MeCP2 and DGGE (denaturing gradient gel electrophoresis) method [Proc. Natl. Acad. Sci. USA, 96, 2913-2018 (1999)] and the like are used. An example is described below.

According to Proc. Natl. Acad. Sci. USA, 89, 1827-1831 (1992), the genome DNA is treated by sodium bisulfite, by which treatment cytosine residues are deaminated into uracil residues while methylated cytosine residues remain as they are.

Primers are designed to amplify the region involved in EGF-R gene expression by polymerase chain reaction (PCR). The primers are designed by hypothesizing a

nucleotide sequence where cytosine is replaced by uracil in the region to be amplified. As the region to be amplified, a part with an abundance of CpG nucleotide sequence is preferable. In EGF-R gene, the promoter region is a preferable example. The example is the sequence from 381st nucleotide to 962nd nucleotide in the nucleotide sequence as described in Seq. ID No. 4. 1114th nucleotide in the nucleotide sequence as described Seq. ID No. 4 corresponds to the translation initiation point of EGF-R, and the region from -152nd nucleotide to -733rd nucleotide upstream therefrom corresponds to the promoter region. Any primer is feasible if it is designed based on the part of nucleotide sequence to be amplified. The examples are nucleotide sequences represented by Seq. ID No. 1 and Seq. ID No.2.

When PCR is performed with the above-mentioned primer, methylated cytosine residues are amplified as cytosines, while unmethylated cytosine residues are amplified as thymine residues. Therefore, the methylation level of cytosine residues in original genome is determined by decoding nucleotide sequence of DNA amplified by said PCR (hereinafter "PCR product").

Decoding nucleotide sequence of said PCR product is carried out as follows:

Firstly said PCR product is fractionated, extracted and purified by agarose gel electrophoresis and the like described in J. Sambrook, E. F. Fritsch, T. Maniatis, Molecular Cloning a Laboratory Manual 2nd Ed., Cold Spring Harbor Laboratory Press (1989) (hereinafter "Molecular Cloning 2nd Ed.").

Then nucleotide sequence determination reaction is performed by using said PCR product as template DNA, primer represented by Seq. ID No. 2, DNA polymerase and dideoxynucleotide. The nucleotide sequence determination reaction is performed by polymerizing nucleotide by DNA polymerase in the presence of dideoxynucleotide. Examples include a method of polymerizing RI-labeled nucleotide as described in Molecular Cloning 2nd Ed. and a method of polymerizing fluorescence-labeled nucleotide by Dye Terminator Cycle Sequencing Kit of Perkin Elmer and Thermal Cycler™ of PerkinElmer.

As methods for decoding nucleotide sequence of the sample after the sequence determination reaction, the method by subjecting the reaction sample having incorporated RI-labeled nucleotide to denatured polyacrylamide gel electrophoresis followed by autoradiography (Molecular Cloning 2nd Ed.), or the method by subjecting the reaction sample having incorporated fluorescence-labeled nucleotide to automatic nucleotide sequence decoding apparatus like PRISM™ 310 Genetic Analyzer (PE Applied Biotechnologies), and the like.

When nucleotide sequence of PCR product is decoded by the above-mentioned process,

in the region of genome DNA where cytosine residue(s) is methylated, the signal(s) indicated by autoradiography or automatic nucleotide sequence decoding apparatus is only a peak(s) of cytosine, while in the region where unmethylated, the signal(s) is only a peak(s) of thymine. However where partially methylated, the signal is a mixture of cytosine and thymine.

To further investigate the methylation level, said PCR product is cloned in a suitable vector and nucleotide sequences of several clones are decoded.

Examples of cloning vectors are plasmid vectors like pUC19 and pBlueScript SK(-) (Stratagene), and phage vectors like M13mp19 and λ gt11.

Cloning is performed according to a method described in Molecular Cloning 2nd Ed. PCR product is inserted and connected into a vector. The vector is transformed into host E. coli like JM109 strain and DH5α strain, and colonies are selected. E. coli of each colony is cultivated and each cloned DNA is extracted and purified.

Nucleotide sequences of thus cloned DNAs are decoded by the method mentioned above. The sequence determination reaction is performed using each of cloned DNAs as template and the nucleotide sequence of reaction sample is decoded by autoradiography or automatic nucleotide sequence decoding apparatus.

Methylation pattern of cytosine residue(s) is analyzed after decoding nucleotide sequence. Specifically, -446th, -443rd, -427th and -417th cytosine residues (corresponding to 668th, 671st, 687th and 697th positions in the nucleotide sequence as described in Seq. ID No. 4) counted from the translation initiation point of EGF-R gene (corresponding to nucleotide number 1114th in the nucleotide sequence as described in Seq. ID No. 4) are analyzed. As understood from the result of diagnosis of 30 patients and 30 healthy people shown in Table 1 of Example 1(10), when the number of methylated residues among 4 residues is not more than 2, the person is diagnosed as psoriasis at the probability of about 83% (25/30). The probability of mistaking healthy person for a patient is about 13%(4/30). When -446th cytosine residue is not methylated, the person of sample is diagnosed as psoriasis at a probability of about 83%(25/30) as shown in Example 1(11). The probability of mistaking healthy person for a patient is about 33%(10/30).

Diagnosis of chronic rheumatoid arthritis is performed in a similar way as diagnosis of psoriasis descried in the above. As understood from the result of diagnosis of 9 patients and 3 healthy people shown in Table 2 of Example 2(9), when the total number of methylated residues among -380th, -372nd and -360th cytosine residues (corresponding to 268th, 276th and 288th positions in the nucleotide sequence as described in Seq. ID No. 8) upstream from translation initiation point of erbB2 gene is not more than 1, the

person of sample is diagnosed as chronic rheumatoid arthritis. The probability of mistaking healthy person for a patient is about 33%(1/3). When -380th cytosine residue (corresponding to 268th position in the nucleotide sequence as described in Seq. ID No. 8) is not methylated, the person of sample is diagnosed as chronic rheumatoid arthritis at a probability of about 67%(6/9) as shown in Example 2(10).

Brief description of Drawinds

Figure 1 shows the state of methylation of -446th, -443rd, -427th and -417th cytosine residues (corresponding to 668th, 671st, 687th and 697th positions in the nucleotide sequence as described in Seq. ID No. 4) from translation initiation point (1114th position in the nucleotide sequence as described in Seq. ID No. 4) of EGF-R genome DNA. N1 to N30 indicates subjects of healthy people and P1 to P30 indicates subjects of psoriasis respectively. Most frequently detected methylation pattern among 20 clones, nucleotide sequences of which were analyzed, is shown as a representative. The case where cytosine residue is methylated is shown by black spot (●).

Figure 2 shows the state of methylation of -380th, -372nd and -360th cytosine residues (corresponding to 268th, 276th and 288th positions in the nucleotide sequence as described in Seq. ID No. 8) upstream from translation initiation point, in the methylation pattern of cytosines in the promoter region of erbB2 gene of genome DNA of samples, obtained as a result of Example 2(7). C1 to C3 indicates 3 subjects of healthy people and R1 to R9 indicates 9 subjects of chronic rheumatoid arthritis respectively. Most frequently detected methylation pattern among 20 clones, nucleotide sequences of which were analyzed, is shown as a representative. The case where cytosine residue is methylated is shown by black spot (●).

Best mode to work the invention

Example 1

(1) Selection of trial subjects

Contraction/non-contraction and the degree of psoriasis with trial subjects were diagnosed by specialized doctors according to the international diagnosis standard (PASI) [Dermatologica, 157, 238-244 (1978), J. Dermatol. Sci., 16, 165-169 (1998)]. Thirty (30) patients diagnosed as psoriasis and 30 healthy people were selected as trial subjects.

(2) Collection of genome DNA from trial subjects

0.4 ml venous blood was taken from the upper arm of each trial subject. DNA was extracted from the blood and purified. Extraction and purification of the DNA were performed by ReadyAmp™ Genomic Purification System (Promega) according to the

attached manual. Purified DNA was dissolved in TE buffer (10mM Tris hydrochloride, 1mM EDTA, pH8.0) to make the final concentration at 100 μg/ml. Thus obtained materials were used as genome DNA.

(3) Sodium bisulfite treatment of genome DNA

According to a method described in Proc. Natl. Acad. Sci. USA, 89, 1827-1832 (1992), each genome DNA obtained in Example 1(2) was treated with sodium bisulfite.

A mixture of 1 μg of genome DNA sample and 9 μg of pBlueScript SK(-) plasmid DNA (Stratagene) as a carrier DNA was incubated in 50 μl of 0.25M sodium hydroxide aqueous solution at 37°C for 10 minutes to be denatured. Thus denatured mixture was mixed with 520 μl of 3.6M sodium bisulfite aqueous solution (pH5.0) and 30 μl of 10mM hydroquinone aqueous solution and incubated at 50°C for 16 hours.

Thus obtained DNA was purified with Wizard Genomic DNA Purification System (Promega) according to the attached manual and the purified DNA was eluted with 50 μl of TE buffer solution (10mM Tris hydrochloride, 1mM EDTA, pH8.0). Eluted DNA was mixed with 5 μl of 2N sodium hydroxide and incubated at a room temperature for 5 minutes. The mixture was mixed/stirred with 100 μl of 100% ethanol and kept at -20°C for 30 minutes. Then the mixture was centrifuged at 10,000×g for 10 minutes to isolate the precipitate. Thus isolated precipitate was suspended in 100 μl of 70% ethanol. The suspension was centrifuged at 10,000×g for 5 minutes to isolate precipitate. Thus isolated precipitate was dried in vacuum and dissolved in 50 μl of pure water to prepare DNA sample treated with sodium bisulfite.

(4) Design and synthesis of PCR primer

The methylation level of cytosine residues of CpG sequence abundant in the region around the promoter of human epidermal growth factor receptor (EGF-R) gene was analyzed. The region around the promoter of EGF-R gene means the region from -152nd nucleotide to -733rd nucleotide upstream form translation initiation point of EGF-R gene, which corresponds to nucleotide sequence from 381st position to 962nd position in the nucleotide sequence as described in Seq. ID No. 4. MP3 (Seq. ID No. 1) and MP4 (Seq. ID No. 2), which are PCR primers for analyzing methylation of cytosine residues of said region, were designed as follows.

Unmethylated cytosine residues are altered to uracil residues by sodium bisulfite treatment described in Example 1(3). Therefore they become thymine residues by subsequent PCR amplification. Seq. ID No. 3 was first designed by hypothesizing the sequence of the region where cytosine residues (C) are replaced by thymine residues (T).

MP4 primer (Seq. ID No. 2) is a sense primer corresponding to the nucleotide sequence from 1st position to 32nd position in Seq. ID No. 3, in which an incision sequence of

restriction enzyme HindIII (AAGCTT) has been introduced into around 5' terminal (from 7th nucleotide to 12th nucleotide in Seq. ID No. 2) to make the subsequent cloning step easy.

MP3 primer (Seq. ID No. 1) is an antisense primer corresponding to the part from 553th nucleotide to 582nd nucleotide in Seq. ID No. 3, in which an incision sequence of restriction enzyme EcoRI (GAATTC) has been introduced into around 5' terminal (from 8th nucleotide to 13th nucleotide in Seq. ID No. 1) to make the subsequence cloning step easy.

Above-mentioned primers were prepared by chemical synthesis of oligonucleotide.

(5) Amplification by PCR

Following PCR was performed by using as template each DNA sample treated by sodium bisulfite (30 derived from patients and 30 derived from healthy people) described in Example 1(3) with MP3 primer and MP4 primer.

Each 1 μl of template DNA was mixed with 35.5 μl of pure water, 5 μl of 10×PCR buffer containing magnesium chloride (GIBCO BRL), 1 μl of 10mM dNTP mix (GIBCO BRL) and 2.5 μl of dimethylsulfoxide (DMSO; Sigma). It was further mixed with 2.5 μl of MP3 primer and MP4 primer solutions in pure water at a concentration of 20 μM each. The mixture (50 μl) was heated at 95°C for 5 minutes and mixed with 1 unit of Taq DNA polymerase (GIBCO BRL). PCR of 35 cycles was performed using Thermal Cycler™ of PerkinElmer with a program of at 96°C for 45 seconds, at 53°C for 30 seconds, at 72°C for 1 minute.

(6) Purification of PCR product

Each PCR product obtained in Example 1(5) was fractionated by low melting point agarose gel electrophoresis according to a method described in Molecular Cloning 2nd Ed. After staining said low melting point agarose gel with ethidium bromide, the part of 582bp (base pair) band of low melting point agarose gel was excised under radiation of ultraviolet light. DNA of the excised band was purified by Wizard PCR Preps DNA Purification System (Promega) according to the attached manual and the purified DNA was eluted with 50 μl of TE buffer (10mM Tris hydrochloride, 1mM EDTA, pH8.0) to obtain purified PCR product.

(7) Direct determination of nucleotide sequence of PCR product

Nucleotide sequences of purified PCR products obtained in Example1(6) were determined.

Firstly sequence determination reaction was performed by Dye Terminator Cycle Sequencing Kit (Perkin Elmer) and Thermal Cycler™ (PerkinElmer), using purified PCR product as template and MP4 primer. During the sequence determination reaction

fluorescence-labeled nucleotide was incorporated. The reaction was performed according to the attached manual.

Then nucleotide sequence of each reaction sample was decoded by using automatic nucleotide sequence decoding apparatus PRISM™310 Genetic Analyzer (PE Applied Biotechnologies) according to the attached manual.

The methylation level of cytosine residues in genome DNA samples taken from each trial subject was determined from the decoded nucleotide sequence. Where cytosine was methylated, signals of automatic nucleotide sequence decoding apparatus were peaks only of cytosine, while where not methylated, signals were only peaks of thymine. However where partially methylated, signals were mixtures of cytosine and thymine.

To further investigate the methylation level, said PCR product was cloned in a vector and nucleotide sequences of several clones were decoded.

(8) Cloning of PCR product and determination of nucleotide sequence

To 10 μl of purified PCR product obtained in Example 1(6), 1 μl of react buffer 2 (GIBCO BRL), 1 unit of restriction enzyme EcoRI (GIBCO BRL) and 1 unit of HindIII (GIBCO BRL) was added, and reacted at 37°C for 1 hour to cleave DNA of purified PCR product. The DNA was mixed/stirred with 100 μl of 100% ethanol, kept at -20°C for 30 minutes and centrifuged at 10,000×g for 10 minutes to obtain precipitate. The precipitate was suspended in 100 μl of 70% ethanol and centrifuged at 10,000×g for 5 minutes to obtain precipitate. The precipitate was dried under vacuum and dissolved in 20 μl of pure water to prepare EcoRI-HindIII cleavage DNA fragment.

In a similar manner 0.5 μg of pBlueScript SK(-)plasmid DNA (Stratagene) was cleaved by restriction enzymes EcoRI and HindIII, DNA fragment of about 2.7kbp was fractionated by low melting point agarose gel electrophoresis described in Molecular Cloning 2nd Ed., the fractionated DNA was purified with Wizard PCR Preps DNA Purification System (Promega) according to the attached manual and the purified DNA was eluted by 50 μl of TE buffer (10mM Tris hydrochloride, 1mM EDTA, pH8.0) to prepare EcoRI-HindIII cleavage vector DNA fragment.

1 μl of thus obtained EcoRI-HindIII cleavage DNA fragment and 0.5 μl of EcoRI-HindIII cleavage vector DNA fragment were mixed. The mixture was mixed with 6 μl of pure water, 2 μl of buffer for T4DNA ligase (GIBCO BRL) and 1 unit of T4DNA ligase (GIBCO BRL), and kept at 16°C for 16 hours to perform DNA ligation reaction.

Each DNA ligation reaction product was transformed into E. coli JM109 strain according to the method described in Molecular Cloning 2nd Ed. Transformed E. coli JM109 strain was cultured on LB plate agar medium (Molecular Cloning 2nd Ed.) containing 50 μg/ml of ampicillin (Sigma), 40 μg/ml of isopropylthio-β-D-galactoside

(Sigma) and 40 μ g/ml of X-gal (Sigma) at 37°C for 1 day. Twenty (20) white colonies were randomly selected from colonies appeared on each plate agar medium and plasmid DNA from each colony was prepared according to Molecular Cloning 2nd Ed.

Using each plasmid DNA as template, universal primer as primer and Dye Terminator Cycle Sequencing Kit (Perkin Elmer) and Thermal Cycler™ (PerkinElmer), the sequence determination reaction was performed, during which reaction fluorescence-labeled nucleotide was incorporated. The reaction was manipulated according to the attached manual. Nucleotide sequence of each reaction sample was decoded by automatic nucleotide sequence decoding apparatus PRISM™ 310 Genetic Analyzer (PE Applied Biotechnologies) according to the attached manual.

The methylation patterns of cytosine residues of genome DNA samples taken from each of trial subjects were determined from the result of decoding.

(9) State of methylation of cytosine residues

Based on the results of Example 1(7) and 1(8), as for methylation pattern of the promoter region of EGF-R gene of genome DNA of trial subjects, methylation of -446th, -443rd, -427th and -417th cytosine residues (668th, 671st, 687th and 697th position in the nucleotide sequence as described in Seq. ID No. 4) is shown in Figure 1. N1 to N30 indicates 30 subjects of healthy people and P1 to P30 indicates 30 subjects of psoriasis patients. Methylation pattern which is most frequently detected in 20 clones, sequences of which were decoded, is shown as a representative. The case where cytosine residue was methylated is represented by black spot (●) while the case where not methylated is left blank.

(10) Diagnostic method for psoriasis (No. 1)

The number of methylated cytosine residues among four of -446th, -443rd, -427th and -417th cytosine residues from transcription initiation point of EGF-R gene was counted. For example, four were found in N1, three in N2, one in P1 and zero in P2. The frequency distribution is tabled in groups of patients and healthy people respectively.

Table 1

Number of methylated residue	0	1	2	3	4
Healthy people	1	0	3	16	10
Patients	15	7	3	5	0

When diagnosis standard is set at the point that the number of methylated residue is not more than 1, psoriasis is diagnosed at a probability of about 73% (22/30). The probability of mistaking a healthy person for a patient is about 3% (1/30).

When diagnosis standard is set at the point that the number of methylated residue is not more than 2, psoriasis is diagnosed at a probability of about 83% (25/30). The

probability of mistaking a healthy person for a patient is about 13% (4/30).

(11) Diagnosis method for psoriasis (No. 2)

-446th cytosine residue is focused on. The residue is methylated in 5 patients and in 20 healthy people. Therefore, when the residue is not methylated, it is diagnosed to be psoriasis at a probability of about 83% (25/30). The probability of mistaking a healthy person for a patient is about 33% (10/30).

Example 2

(1) Selection of trial subjects

Contraction/non-contraction and the degree of chronic rheumatoid arthritis with trial subjects were diagnosed by specialized doctors according to the diagnosis standard of American Rheumatoid Academy [Arthritis Reum., 31, 315-324 (1988)]. Nine (9) patients diagnosed as chronic rheumatoid arthritis and 3 healthy people were selected as trial subjects.

(2) Collection of genome DNA from trial subjects

0.4ml venous blood was taken from the upper arm of each trial subject of Example 2(1). DNA was extracted from the taken blood and purified. Extraction and purification of the DNA was performed by ReadyAmp™ Genomic DNA Purification System (Promega) according to the attached manual. Purified DNA was dissolved in TE buffer (10mM Tris hydrochloride, 1mM EDTA, pH8.0) to make the final concentration at 100 μg/ml. Thus obtained materials were used as genome DNA.

(3) Sodium bisulfite treatment of genome DNA samples

According to a method described in Proc. Natl. Acad. Sci. USA, 89, 1827-1832 (1992) each genome DNA obtained in Example 2(2) was treated with sodium bisulfite.

A mixture of 1 μg of genome DNA and 9 μg of pBlueScript SK(-) plasmid DNA (Stratagene) as a carrier DNA was added to 50 μl of 0.25M sodium hydroxide aqueous solution and incubated at 37°C for 10 minutes to be denatured. Thus denatured mixture was mixed with 520 μl of 3.6M sodium bisulfite aqueous solution (pH5.0) and 30 μl of 10mM hydroquinone aqueous solution and incubated at 50°C for 16 hours.

Thus obtained DNA was purified with Wizard Genomic DNA Purification System (Promega) according to the attached manual and the purified DNA was eluted with 50 μl of TE buffer solution (10mM Tris hydrochloride, 1mM EDTA, ph8.0). Eluted DNA was mixed with 5 μl of 2N sodium hydroxide and incubated at a room temperature for 5 minutes. The mixture was mixed/stirred with 100 μl of 100% ethanol and kept at -20°C for 30 minutes. Then the mixture was centrifuged at 10,000×g for 10 minutes to isolate the precipitate. Thus obtained precipitate was suspended in 100 μl of 70% ethanol. The suspension was subjected to centrifugation at 10,000×g for 5 minutes to obtain

precipitate. Thus obtained precipitate was dried in vacuum and dissolved in 50 μl of pure water to prepare DNA sample treated with sodium bisulfite.

(4) Design and synthesis of PCR primer

The methylation level of cytosine residues of CpG sequence abundant in the region around the promoter of human epidermal growth factor-like receptor 2 (erbB2/HER2/neu) gene (hereinafter "erbB2 gene") was analyzed. The region around the promoter of erbB2 gene means the region around the nucleotide sequence from -1st position to -647th position upstream from translation initiation point of erbB2 gene, concretely describing, the region around the nucleotide sequence represented by Seq. ID No. 8 [Mol. Cell. Biol., 7, 2597-2601 (1987), Gene 136, 361-364 (1993)]. This corresponds to the nucleotide sequence from 3001st position to 3647th position in the nucleotide sequence represented by accession number Z13970 of GenBank database. In Seq. ID No. 8, the nucleotide sequence from 87th position to 449th position was focused on in this example. This region corresponds to the nucleotide sequence from -199th to -561st upstream from translation initiation point. NeuS (Seq. ID No. 5) and NeuA (Seq. ID No. 6), which are PCR primers for analyzing methylation of cytosine residues of said region, were designed as follows.

Seq. ID No. 7 was designed by hypothesizing the nucleotide sequence where cytosine residues (C) in the region (nucleotide sequence from 87th position to 449th position in Seq. ID No. 8) were replaced by thymine residues (T), because unmethylated cytosine residues are thought to be altered to uracil residues by sodium bisulfite treatment described in Example 2(3) and nucleotide sequences after PCR amplification are thought to be altered to thymine residues.

NeuS primer (Seq. ID No. 5) is a sense primer corresponding to the nucleotide sequence from 1st position to 25th position of Seq. ID No. 7. NeuA primer (Seq. ID No. 6) is an antisense primer corresponding to the nucleotide sequence from 339th to 363rd of Seq. ID No. 7. Chemical synthesis of those primer oligonucleotides was performed by Phosphoramidite method [S. L. Beaucage & M. H. Caruthers, Tetrahedron Letters, 22, 1859 (1981)].

(5) Amplification by PCR

Using each DNA sample treated with sodium bisulfite described in Example 2(3) as template DNA (9 derived from patients and 3 derived from healthy people) and NeuS primer and NeuA primer, PCR was performed as follows:

Each 1 μl of template DNA was mixed with 35.5 μl of pure water, 5 μl of 10×PCR buffer containing magnesium chloride (GIBCO BRL), 1 μl of 10mMdNTP mix (GIBCO BRL) and 2.5 μl of dimethylsulfoxide (DMSO; Sigma). It was further mixed with each

2.5 μl of NeuS primer and NeuA primer dissolved in pure water at a concentration of 20 μM. The mixture (50 μl) was heated at 95°C for 5 minutes and mixed with 1 unit of Taq DNA polymerase (GIBCO BRL). PCR of 40 cycles was performed using Thermal Cycler™ of PerkinElmer with a program of at 94°C for 30 seconds, at 53°C for 30 seconds, at 72°C for 1 minute.

(6) Purification of PCR product

Each PCR product obtained in Example 2(5) (9 samples collected from patients and 3 samples collected from healthy people) was fractionated by low melting point agarose gel electrophoresis according to the method described in Molecular Cloning 2nd Ed. After staining said low melting point agarose gel with ethidium bromide, the part of 363bp (base pair) band of low melting point agarose gel was excised under radiation of ultraviolet light. DNA of the excised part was purified by Wizard PCR Preps DNA Purification System (Promega) according to the attached manual and the purified DNA was eluted with 50 μl of TE buffer (10mM Tris hydrochloride, 1mM EDTA, pH8.0) to obtain purified PCR product.

(7) Cloning of PCR product and nucleotide sequence determination

Each 10 μl of purified PCR products obtained in Example 2(6) (9 samples collected from patients and 3 samples collected from healthy people) was subjected to cloning by using commercially available TA cloning kit pGEM-T Easy Vector System II (Promega) according to the attached protocol (Promega Protocol TM042). Finally transformed E. coli JM109 strain (attached to the kit) was cultured on LB plate agar medium (Molecular Cloning 2nd Ed.) containing 50 μg/ml of ampicillin (Sigma), 40 μg/ml of isopropylthio-β-D-galactoside (Sigma) and 40 μg/ml of X-gal (Sigma) at 37°C for 1 day. Twenty (20) white colonies were randomly selected from colonies appeared on each plate agar medium and plasmid DNA was prepared from each colony according to Molecular Cloning 2nd Ed.

Using each plasmid DNA as template, Universal Primer as primer and Dye Terminator Cycle Sequencing Kit (Perkin Elmer) and Thermal Cycler™ (PerkinElmer), sequence determination reaction was performed, during which reaction fluorescence-labeled nucleotide was incorporated. The reaction was manipulated according to the manual attached to the kit. Nucleotide sequence of each reaction sample was decoded by automatic nucleotide sequence decoding apparatus PRISM™ 310 Genetic Analyzer (PE Applied Biotechnologies) according to the manual attached to the apparatus.

The methylation patterns of cytosine residues in genome DNA samples taken from trial subjects were determined from the results of decoding. Namely, cytosine methylated in genome was read as cytosine by the automatic nucleotide sequence

decoding apparatus, while unmethylated cytosine was read as thymine.

(8) State of methylation of cytosine residues

Based on the results of Example 2(7), as for methylation pattern of promoter region of erbB2 gene of genome DNA of trial subjects, the state of methylation of -380th, -372nd and -360th cytosine residues (corresponding to base number 268th, 276th and 288th position in the nucleotide sequence as described in Seq. ID No. 8) is shown in Figure 2. C1 to C3 indicates 3 subjects of healthy people and R1 to R9 indicates 9 subjects of chronic rheumatoid arthritis patients. Methylation pattern which was most frequently detected in 20 clones, sequences of which were decoded, is shown as a representative. The case where cytosine residue was methylated is represented by black spot (●) while the case where not methylated is left blank.

(9) Diagnostic method for chronic rheumatoid arthritis (No. 1)

As to the number of methylated cytosine residues among -380th, -372nd and -360th cytosine residues from transcription initiation point of erbB2 gene, the frequency distribution is tabled below in groups of patients and healthy people respectively.

Table 2

Number of methylated residue	0	1	2
Healthy people	0	1	2
Patients	6	3	0

When the number of methylated residue is not more than 1, chronic rheumatoid arthritis is diagnosed. The probability of mistaking a healthy person for a patient is about 33% (1/3).

(10) Diagnosis method for chronic rheumatoid arthritis (No. 2)

Cytosine residue at nucleotide number 268 is focused on. The residue was methylated in all 3 healthy people while in only 3 of 9 patients. Therefore, when the cytosine residue is not methylated, it is diagnosed to be chronic rheumatoid arthritis at a probability of about 67% (6/9).

Industrial Applicability

A diagnostic method for detecting cell-proliferating diseases by extracting genome DNA from blood etc. of trial subject and analyzing the methylation level of cytosine residues of cell-proliferating factor receptor gene is provided.

Claims

1, A diagnostic method for detecting cell-proliferating diseases characterized by determining the methylation level of cytosine residue(s) at the specific region of genome

DNA involved in the expression of cytokine receptor gene.

2, A diagnostic method in claim 1 wherein the cytokine receptor gene is the gene of a receptor selected from tyrosine kinase receptor family, serine-threonine kinase receptor family, interleukin receptor family, interferon receptor family, immunoglobulin receptor family, apoptotic receptor family and seven transmembrane receptor family.

3, A diagnostic method in claim 2 wherein the tyrosine kinase receptor family gene is the gene of a receptor selected form epidermal growth factor receptor, epidermal growth factor-like receptor 2 (erbB2/HER2/neu), platelet derived growth factor receptor and vascular endothelial cell growth factor receptor.

4, A diagnostic method in claim 1 wherein the cell-proliferating disease is a cell-proliferating disease selected from psoriasis, chronic rheumatoid arthritis, arteriosclerosis, restenosis, diabetic retinopathy, retinopathy of prematurity and solid tumor.

5, A diagnostic method in claim 1 wherein the specific region is a region in CpG island of promoter or intron.

6, A diagnostic method in claim 1 wherein the specific region is a region involved in the expression of epidermal growth factor receptor gene and a region represented by the nucleotide sequence from 381st position to 962nd position in the nucleotide sequence as described in Seq. ID No. 4.

7, A diagnostic method in claim 6 characterized by determining the methylation level of 668th, 671st, 687th and 697th cytosine residues in the nucleotide sequence as described in Seq. ID No. 4.

8, A diagnostic method in claim 7 characterized by analyzing the methylation level of 668th cytosine residue in the nucleotide sequence as described in Seq. ID No. 4.

9, A DNA primer having base sequence represented by Seq. ID No. 1 or 2 used for any one of diagnosis described in claims 1 to 8.

10, A diagnostic method in claim 1 wherein the specific region is the region involved in the expression of epidermal growth factor-like receptor 2 (erbB2/HER2/neu) and represented by the nucleotide sequence of Seq. ID No. 8.

11, A diagnostic method in claim 10 characterized by determining the level of methylation of 268th, 276th and 288th cytosine residues in the nucleotide sequence as described in Seq. ID No. 8.

12, A diagnostic method in claim 11 characterized by analyzing the level of methylation of 268th cytosine residue in the nucleotide sequence as described in Seq. ID No. 8.

13, A DNA primer having base sequence represented by Seq. ID No. 5 or 6 used for any one of diagnosis in claims 1 to 4 and 10 to 12.

14, A DNA having base sequence represented by Seq. ID No. 1, 2, 5 or 6.

15, A method of detecting the level of methylation of cytosine residue(s) in the specific region of DNA involved in the expression of cytokine receptor gene.

16, A method in claim 15 wherein the method of detecting the level of methylation is a method using methylation sensitive restriction enzyme, a method using chemical modification by hydrazine, permanganic acids or sodium bisulfite, an immunological method using antibodies specific to methylated DNA, affinity column chromatography method or DGGE (denaturing gradient gel electrophoresis) method.

Abstract

This invention relates to a diagnostic method for detecting cell-proliferating diseases characterized by analysis of the methylation level of cytosine residues in the region involved in the expression of cytokine receptor gene.

PCT09

CRF Errors Corrected by the STIC Systems Branch

Serial Number: 09/787,292

CRF Processing Date: _____
Edited by: _____
Verified by: _____ (STIC sta

ENTERED

- ☐ Changed a file from non-ASCII to ASCII
- ☐ Changed the margins in cases where the sequence text was "wrapped" down to the next line.
- ☐ Edited a format error in the Current Application Data section, specifically: _____
- ☐ Edited the Current Application Data section with the actual current number. The number inputted by the applicant was ☐ the prior application data; or ☐ other _____.
- ☐ Added the mandatory heading and subheadings for "Current Application Data".
- ☐ Edited the "Number of Sequences" field. The applicant spelled out a number instead of using an integer.
- ☐ Changed the spelling of a mandatory field (the headings or subheadings), specifically: _____
- ☐ Corrected the SEQ ID NO when obviously incorrect. The sequence numbers that were edited were: _____
- ☐ Inserted or corrected a nucleic number at the end of a nucleic line. SEQ ID NO's edited: _____
- ☐ Corrected subheading placement. All responses must be on the same line as each subheading. If the applicant placed a response below the subheading, this was moved to its appropriate place.
- ☐ Inserted colons after headings/subheadings. Headings edited included: _____
- ☐ Deleted extra, invalid, headings used by an applicant, specifically: _____
- ☐ Deleted: ☐ non-ASCII "garbage" at the beginning/end of files; ☐ secretary initials/filename at end of file; ☐ page numbers throughout text; ☐ other invalid text, such as _____.
- ☐ Inserted mandatory headings, specifically: _____
- ☐ Corrected an obvious error in the response, specifically: _____
- ☐ Edited identifiers where upper case is used but lower case is required, or vice versa.
- ☐ Corrected an error in the Number of Sequences field, specifically: _____
- ☐ A "Hard Page Break" code was inserted by the applicant. All occurrences had to be deleted.
- ☐ Deleted *ending* stop codon in amino acid sequences and adjusted the "(A)Length:" field accordingly (error due to a PatentIn bug). Sequences corrected: _____
- ☑ Other: Corrected "end of line" nucleic number. Sequence #4.

*Examiner: The above corrections must be communicated to the applicant in the first Office Action. DO NOT send a copy of this form.

3/1/95

RAW SEQUENCE LISTING DATE: 06/06/2001
PATENT APPLICATION: US/09/787,292 TIME: 17:50:59

Input Set : A:\Cpg.pto
Output Set: C:\CRF3\06062001\I787292.raw

<110> APPLICANT: Homma, Yoshimi
Oyama, Noritaka
Sato, Koichiro
<120> TITLE OF INVENTION: Method for diagnosis of cell proliferative disease
<130> FILE REFERENCE: 31761-169944
<140> CURRENT APPLICATION NUMBER: US 09/787,292
C--> 13 **<141> CURRENT FILING DATE: 2001-05-09**
<150> PRIOR APPLICATION NUMBER: PCT/JP99/05069
<151> PRIOR FILING DATE: 1999-09-17
<160> NUMBER OF SEQ ID NOS: 8
<170> SOFTWARE: PatentIn Ver. 2.0
<210> SEQ ID NO: 1
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:Synthetic DNA
<400> SEQUENCE: 1
ccaaaacgaa ttcaaaactc caaccacctc 30
<210> SEQ ID NO: 2
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:Synthetic DNA
<400> SEQUENCE: 2
taggggaagc ttggggattt gaataaagga gt 32
<210> SEQ ID NO: 3
<211> LENGTH: 582
<212> TYPE: DNA
<213> ORGANISM: Human
<400> SEQUENCE: 3
taggggggagg ttggggattt gaataaagga gtagtttttt tgttggtgtt attatttgat 60
gttggtttta aggtttggtt agtttgttta aagttggtat aagtttgttt tgtaaaataa 120
aagaagggaa aggggggaagg ggattttggt atagatttgg tttgatttgg atataggttg 180
ggttgtaagt ttgtggggat tgggtttaga ggggtagtgt tgggaatgtt ttttttggaa 240
attaattttt tagggtattg ttttttttttt atgtgttgtt ttatttttgt tggagattag 300
gttttgtggg ggttattgtg tttattgttt tgtggttgtt ggttttgggt ttttgttgtt 360
ggtttttttt ttttttttttt gtatttttttt ttttttttgt ttttttgat ttttttttg 420
ttgtttggtt ttttttttttt ttgttttgtt ttttgtgttt tggtttgtgt gagttagatg 480
tttgggtagt ttttggtgta gtgtggttgt agtagttttt ttttttgta tggtgtgagt 540
gtttgttgtg ttgaggtggt tggagttttg agttagtttt gt 582
<210> SEQ ID NO: 4
<211> LENGTH: 1200
<212> TYPE: DNA
<213> ORGANISM: Human
<400> SEQUENCE: 4

RAW SEQUENCE LISTING DATE: 06/06/2001
PATENT APPLICATION: US/09/787,292 TIME: 17:50:59

Input Set : A:\Cpg.pto
Output Set: C:\CRF3\06062001\I787292.raw

```
 69 aagcttccgc gagtttccca ggcatttctc ctcgcgggac taccaggggt agtgggacac        60
 70 ttagcctctc taaaagcacc tccacggctg tttgtgtcaa gcctttattc caagagcttc       120
 71 acttttgcga agtaatgtgc ttcacacatt ggcttcaaag tacccatggc tggttgcaat       180
 72 aaacattaag gaggcctgtc tctgcacccg gagttggtgc cctcatttca gatgatttcg       240
 73 agggtgcttg acaagatctg aaggaccctc ggactttaga gcaccacctc ggaacgcctg       300
 74 gcaccctctgc cgcgcgggca cggcgacctc ctcagctgcc aggccagcct ctgatccccg       360
 75 cgaggggtcc cgtagtgctg caggggggag ctggggaccc gaataaagga gcagtttccc       420
 76 cgtcggtgcc attatccgac gctggctcta aggctcggcc agtctgtcta aagctggtac       480
 77 aagtttgctt tgtaaaacaa aagaaggga aggggggaagg ggaccctggc acagatttgg       540
 78 ctcgacctgg acataggctg ggctgcaagt ccgcgggcac cgggtccaga ggggcagtgc       600
 79 tgggaacgcc cctctcggaa attaactcct cagggcaccg ctcccctccc atgcgccgcc       660
 80 ccactcccgc cggagactag gtcccgcggg ggccaccgtg tccaccgcct cgcggccgct       720
 81 ggccttgggt ccccgctgct ggttctcctc cctcctcctc gcattctcct cctcctctgc       780
 82 tcctcccgat ccctcctccg ccgcctggtc cctcctcctc ccgccctgcc tcccgcgcct       840
 83 cggcccgcgc gagctagacg tccgggcagc ccccggcgca gcgcggccgc agcagcctcc       900
 84 tccccccgca cggtgtgagc gcccgccgcg ccgaggcggc cggagtcccg agctagcccc       960
 85 gcggccgccg ccgcccagac cggacgacag gccaccctgt cgcgtccgcc cgagtccccg      1020
 86 cctcgccgcc aacgccacaa ccacccgcca cggccccctg actccgtcca gtattgatcg      1080
 87 ggagagccgg agcgagctct tcggggagca gcgatgcgac cctccgggac ggccggggca      1140
 88 gcgctcctgg cgctgctggc tgcgctctgc ccggcgagtc gggctctgga ggaaaagaaa      1200
 90 <210> SEQ ID NO: 5
 91 <211> LENGTH: 25
 92 <212> TYPE: DNA
 93 <213> ORGANISM: Artificial Sequence
 95 <220> FEATURE:
 96 <223> OTHER INFORMATION: Description of Artificial Sequence:Synthetic DNA
 98 <400> SEQUENCE: 5
 99 ggtttgggat ggagtaggat gtaag                                              25
101 <210> SEQ ID NO: 6
102 <211> LENGTH: 25
103 <212> TYPE: DNA
104 <213> ORGANISM: Artificial Sequence
106 <220> FEATURE:
107 <223> OTHER INFORMATION: Description of Artificial Sequence:Synthetic DNA
109 <400> SEQUENCE: 6
110 cttatacttc ctcaaacaac cctcc                                             25
112 <210> SEQ ID NO: 7
113 <211> LENGTH: 363
114 <212> TYPE: DNA
115 <213> ORGANISM: Human
117 <400> SEQUENCE: 7
118 ggtttgggat ggagtaggat gtaagttttt taggaagtta gataattgag atttaaaggg       60
119 tgttaagagt ggtagtttag ggaaatttat tttggatttt gggggagggg gtagagttat      120
120 tagtttttgt atttagggat ttttgagga aaagtgtgag aatggttgta ggtaatttag      180
121 gtgttttggt gttaggaggg atgatttagg tttgtgtgaa gagagggaga aagtgaagtt      240
122 ggagagttgtt gatttttaga ttttgttgga atgtagttgg agggggtgag ttgggagtgt      300
123 gtttgttttt aattattgga gaaggaggag gtggaggagg agggttgttt gaggaagtat      360
124 aag                                                                   363
126 <210> SEQ ID NO: 8
```

RAW SEQUENCE LISTING DATE: 06/06/2001
PATENT APPLICATION: **US/09/787,292** TIME: 17:50:59

Input Set : **A:\Cpg.pto**
Output Set: **C:\CRF3\06062001\I787292.raw**

```
<211> LENGTH: 647
<212> TYPE: DNA
<213> ORGANISM: Human
<400> SEQUENCE: 8
aggtaaacac aacacatccc cctccttgac tatcaatttt actagaggat gtggtgggaa      60
aaccattatt tgatattaaa acaataggct tgggatggag taggatgcaa gctccccagg     120
aagttagata actgagactt aaagggtgtt aagagtggca gcctagggaa atttatcccg     180
gactccgggg gagggggcag agtcaccagc ctctgcattt agggattctc cgaggaaaag     240
tgtgagaacg gctgcaggca acccaggcgt cccggcgcta ggagggacga cccaggcctg     300
cgcgaagaga gggagaaagt gaagctggga gttgccgact cccagacttc gttggaatgc     360
agttggaggg ggcgagctgg gagcgcgctt gctcccaatc accggagaag gaggaggtgg     420
aggaggaggg ctgcttgagg aagtataaga atgaagttgt gaagctgaga ttcccctcca     480
ttgggaccgg agaaaccagg ggagcccccc gggcagccgc gcgccccttc ccacggggcc     540
ctttactgcg ccgcgcgccc ggcccccacc cctcgcagca ccccgcgccc cgcgccctcc     600
cagccgggtc cagccggagc catggggccg gagccgcagt gagcacc                   647
```

VERIFICATION SUMMARY
PATENT APPLICATION: US/09/787,292

DATE: 06/06/2001
TIME: 17:51:00

Input Set : A:\Cpg.pto
Output Set: C:\CRF3\06062001\I787292.raw

L:13 M:271 C: Current Filing Date differs, Replaced Current Filing Date

PCT 09

RAW SEQUENCE LISTING
PATENT APPLICATION: US/09/787,292

DATE: 05/29/2001
TIME: 15:54:50

Input Set : A:\SEQUENCE LISTING txt.txt
Output Set: C:\CRF3\05292001\I787292.raw

Does Not Comply
Corrected Diskette Needed

```
      3 <110> APPLICANT: Homma, Yoshimi
      4       Oyama, Noritaka
      5       Sato, Koichiro
      7 <120> TITLE OF INVENTION: Method for diagnosis of cell proliferative disease
     10 <130> FILE REFERENCE: 31761-169944
     12 <140> CURRENT APPLICATION NUMBER: US 09/787,292
C--> 13 <141> CURRENT FILING DATE: 2001-05-09
     15 <150> PRIOR APPLICATION NUMBER: PCT/JP99/05069
     16 <151> PRIOR FILING DATE: 1999-09-17
     20 <160> NUMBER OF SEQ ID NOS: 8
     22 <170> SOFTWARE: PatentIn Ver. 2.0


ERRORED SEQUENCES

     63 <210> SEQ ID NO: 4
     64 <211> LENGTH: 1200
     65 <212> TYPE: DNA
     66 <213> ORGANISM: Human
     68 <400> SEQUENCE: 4
     69 aagcttccgc gagtttccca ggcatttctc ctcgcgggac taccaggggt agtgggacac      60
     70 ttagcctctc taaaagcacc tccacggctg tttgtgtcaa gcctttattc caagagcttc     120
     71 acttttgcga agtaatgtgc ttcacacatt ggcttcaaag tacccatggc tggttgcaat     180
     72 aaacattaag gaggcctgtc tctgcacccg gagttggtgc cctcatttca gatgatttcg     240
     73 agggtgcttg acaagatctg aaggaccctc ggactttaga gcaccacctc ggaacgcctg     300
     74 gcacccctgc cgcgcgggca cggcgacctc ctcagctgcc aggccagcct ctgatccccg     360
     75 cgaggggtcc cgtagtgctg caggggggagg ctggggaccc gaataaagga gcagtttccc     420
     76 cgtcggtgcc attatccgac gctggctcta aggctcggcc agtctgtcta aagctggtac     480
     77 aagtttgctt tgtaaaacaa aagaagggaa agggggaagg ggaccctggc acagatttgg     540
     78 ctcgacctgg acataggctg ggctgcaagt ccgcggggac cgggtccaga ggggcagtgc     600
     79 tgggaacgcc cctctcggaa attaactcct cagggcaccg ctcccctccc atgcgccgcc     660
     80 ccactcccgc cggagactag gtcccgcggg ggccaccgtg tccaccgcct cgcggccgct     720
     81 ggccttgggt ccccgctgct ggttctcctc cctcctcctc gcattctcct cctcctctgc     780
     82 tcctcccgat ccctcctccg ccgcctggtc cctcctcctc ccgccctgcc tcccgcgcct     840
     83 cggcccgcgc gagctagacg tccgggcagc ccccggcgca gcgcggccgc agcagcctcc     900
     84 tccccccgca cggtgtgagc gcccgccgcg ccgaggcggc cggagtcccg agctagcccc     960
     85 gcggccgccg ccgcccagac cggacgacag gccacctcgt cgcgtccgcc cgagtccccg    1020
     86 cctcgccgcc aacgccacaa ccaccgcgca cggccccctg actccgtcca gtattgatcg    1080
     87 ggagagccgg agcgagctct tcggggagca gcgatgcgac cctccgggac ggccgggaca    1140
E--> 88 gcgctcctgg cgctgctggc tgcgctctgc ccggcgagtc gggctctgga ggaaaagaaa    120
```

/1200 ?

VERIFICATION SUMMARY
PATENT APPLICATION: US/09/787,292

DATE: 05/29/2001
TIME: 15:54:51

Input Set : A:\SEQUENCE LISTING txt.txt
Output Set: C:\CRF3\05292001\I787292.raw

L:13 M:271 C: Current Filing Date differs, Replaced Current Filing Date
L:88 M:254 E: No. of Bases conflict, LENGTH:Input:120 Counted:1200 SEQ:4

SEQUENCE LISTING

<110> Kyowa Hakko Kogyo Co., Ltd

<120> Diagnostic method for detecting cell-proliferating diseases

<130> 11155

<140>
<141>

<150> H10-265089
<151> 1998-09-18

<160> 8

<170> PatentIn Ver. 2.0

<210> 1
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Synthetic DNA

<400> 1
ccaaaacgaa ttcaaaactc caaccacctc 30

<210> 2
<211> 32
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Synthetic DNA

<400> 2
taggggaagc ttggggattt gaataaagga gt 32

<210> 3
<211> 582
<212> DNA
<213> Human

<400> 3
tagggggagg ttggggattt gaataaagga gtagtttttt tgttggtgtt attatttgat 60
gttggtttta aggtttggtt agtttgttta aagttggtat aagtttgttt tgtaaaataa 120
aagaaggaaa agggggaagg ggattttggt atagatttgg tttgatttgg atataggttg 180
ggttgtaagt ttgtggggat tgggtttaga ggggtagtgt tgggaatgtt ttttttggaa 240
attaattttt tagggtattg tttttttttt atgtgttgtt ttatttttgt tggagattag 300
gttttgtggg ggttattgtg tttattgttt tgtggttgtt ggttttgggt ttttgttgtt 360
ggtttttttt ttttttttt gtatttttt ttttttttgt ttttttgat tttttttttg 420
ttgtttggtt ttttttttt ttgttttgtt ttttgtgttt tggtttgtgt gagttagatg 480
tttgggtagt ttttggtgta gtgtggttgt agtagttttt ttttttgta tggtgtgagt 540
gtttgttgtg ttgaggtggt tggagttttg agttagtttt gt 582

<210> 4
<211> 1200
<212> DNA
<213> Human

<400> 4
aagcttccgc gagtttccca ggcatttctc ctcgcgggac taccaggggt agtgggacac 60
ttagcctctc taaaagcacc tccacggctg tttgtgtcaa gcctttattc caagagcttc 120
acttttgcga agtaatgtgc ttcacacatt ggcttcaaag tacccatggc tggttgcaat 180
aaacattaag gaggcctgtc tctgcacccg gagttggtgc cctcatttca gatgatttcg 240
agggtgcttg acaagatctg aaggaccctc ggactttaga gcaccacctc ggaacgcctg 300
gcacccctgc cgcgcgggca cggcgacctc ctcagctgcc aggccagcct ctgatccccg 360
cgagggtcc cgtagtgctg caggggagg ctggggaccc gaataaagga gcagtttccc 420
cgtcggtgcc attatccgac gctggctcta aggctcggcc agtctgtcta aagctggtac 480
aagtttgctt tgtaaaacaa aagaagggaa agggggaagg ggaccctggc acagatttgg 540
ctcgacctgg acataggctg ggctgcaagt ccgcggggac cgggtccaga ggggcagtgc 600

tgggaacgcc cctctcggaa attaactcct cagggcaccg ctcccctccc atgcgccgcc 660
ccactcccgc cggagactag gtcccgcggg ggccaccgtg tccaccgcct cgcggccgct 720
ggccttgggt ccccgctgct ggttctcctc cctcctcctc gcattctcct cctcctctgc 780
tcctcccgat ccctcctccg ccgcctggtc cctcctcctc ccgccctgcc tcccgcgcct 840
cggcccgcgc gagctagacg tccgggcagc ccccggcgca gcgcggccgc agcagcctcc 900
tccccccgca cggtgtgagc gcccgccgcg ccgaggcggc cggagtcccg agctagcccc 960
gcggccgccg ccgcccagac cggacgacag gccacctcgt cgcgtccgcc cgagtccccg 1020
cctcgccgcc aacgccacaa ccaccgcgca cggccccctg actccgtcca gtattgatcg 1080
ggagagccgg agcgagctct tcggggagca gcgatgcgac cctccgggac ggccggggca 1140
gcgctcctgg cgctgctggc tgcgctctgc ccggcgagtc gggctctgga ggaaaagaaa 1200

<210>5
<211>25
<212>DNA
<213>Artificial Sequence

<220>
<223>Description of Artificial Sequence:Synthetic DNA

<400>5
ggtttgggat ggagtaggat gtaag 25

<210>6
<211>25
<212>DNA
<213>Artificial Sequence

<220>
<223>Description of Artificial Sequence:Synthetic DNA

<400>6
cttatacttc ctcaaacaac cctcc 25

<210>7
<211>363
<212>DNA
<213>Human

<400>7

ggtttgggat ggagtaggat gtaagttttt taggaagtta gataattgag atttaaaggg 60
tgttaagagt ggtagtttag ggaaatttat tttggatttt gggggagggg gtagagttat 120
tagtttttgt atttagggat tttttgagga aaagtgtgag aatggttgta ggtaatttag 180
gtgttttggt gttaggaggg atgatttagg tttgtgtgaa gagagggaga aagtgaagtt 240
gggagttgtt gattttaga ttttgttgga atgtagttgg aggggggtgag ttgggagtgt 300
gtttgttttt aattattgga gaaggaggag gtggaggagg agggttgttt gaggaagtat 360
aag 363

<210>8
<211>647
<212>DNA
<213>Human

<400>8

aggtaaacac aacacatccc cctccttgac tatcaatttt actagaggat gtggtgggaa 60
aaccattatt tgatattaaa acaataggct tgggatggag taggatgcaa gctccccagg 120
aagttagata actgagactt aaagggtgtt aagagtggca gcctagggaa atttatcccg 180
gactccgggg gaggggcag agtcaccagc ctctgcattt agggattctc cgaggaaaag 240
tgtgagaacg gctgcaggca acccaggcgt cccggcgcta ggagggacga cccaggcctg 300
cgcgaagaga gggagaaagt gaagctggga gttgccgact cccagacttc gttggaatgc 360
agttggaggg ggcgagctgg gagcgcgctt gctcccaatc accggagaag gaggaggtgg 420
aggaggaggg ctgcttgagg aagtataaga atgaagttgt gaagctgaga ttcccctcca 480
ttgggaccgg agaaaccagg ggagcccccc gggcagccgc gcgccccttc ccacggggcc 540
ctttactgcg ccgcgcgccc ggccccacc cctcgcagca ccccgcgccc cgcgccctcc 600
cagccgggtc cagccggagc catggggccg gagccgcagt gagcacc 647

FIG. 1

Healthy people

	-446	-443	-427	-417
N1	●	●	●	●
N2	●		●	●
N3	●	●	●	●
N4	●	●	●	●
N5	●	●	●	●
N6	●	●		●
N7	●	●	●	
N8	●	●		●
N9		●	●	●
N10	●	●	●	●
N11	●	●		
N12	●	●		
N13		●	●	●
N14	●	●	●	●
N15		●		●
N16	●	●	●	●
N17	●	●	●	●
N18		●	●	●
N19		●	●	●
N20	●	●	●	
N21	●	●	●	●
N22		●	●	●
N23		●	●	●
N24		●	●	●
N25	●	●	●	
N26	●	●		●
N27		●	●	●
N28	●	●	●	●
N29	●	●		●
N30				

Patients of psoriasis

	-446	-443	-427	-417
P1		●		
P2				
P3		●	●	
P4				
P5				
P6				
P7		●		
P8		●	●	●
P9				
P10	●	●		
P11	●			
P12				
P13		●	●	●
P14				
P15				
P16				
P17	●		●	●
P18				●
P19				
P20		●	●	●
P21				
P22				
P23	●	●		
P24		●		
P25				
P26	●		●	●
P27				
P28		●		
P29				●
P30				

FIG. 2

Healthy people

	-380	-372	-360
C1	●		●
C2	●		
C3	●	●	

Patients of chronic rheumatoid arthritis

	-380	-372	-360
R1	●		
R2			
R3			
R4	●		
R5			
R6			
R7			
R8			
R9	●		

DECLARATION FOR UNITED STATES PATENT APPLICATION
POWER OF ATTORNEY, DESIGNATION OF CORRESPONDENCE ADDRESS

Attorney Docket
31671-169944 RK

Page 1 of 2

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and that I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHOD FOR THE DIAGNOSIS OF CELL PROLIFERATIVE DISEASE, the specification of which

[ ] is attached hereto.
[ ] was filed on _____, as Application No. _____, and was amended on _____ [if applicable].
[X] was filed under the Patent Cooperation Treaty on September 17, 1999, Serial No. PCT/JP99/05069, the United States of America being designated.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent, utility model, design or inventor's certificate listed below and have also identified below any foreign application(s) for patent, utility model, design or inventor's certificate having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
Number	Country	Date Filed	Yes	No
10-265089	Japan	September 18, 1998	X	

I hereby appoint the following attorneys and agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: George H. Spencer (Reg. No. 18,038), Richard L. Aitken, (Reg. No. 18,791), Robert J. Frank (Reg. No. 19,112), Norman N. Kunitz (Reg. No. 20,586), Gabor J. Kelemen (Reg. No. 21,016), Laurence J. Marhoefer, (Reg. No. 21,091), Marina V. Schneller (Reg. No. 26,032), Robert Kinberg (Reg. No. 26,924), Allen Wood (Reg. No. 28,134), John P. Shannon, (Reg. No. 29,276), Clifton E. McCann, (Reg. No. 29,565), Ashley J. Wells (Reg. No. 29,847), Richard D. Schmidt (Reg. No. 31,301), James R. Burdett (Reg. No. 31,594), Michael A. Gollin (Reg. No. 31,957), Catherine M. Voorhees (Reg. No. 33,074), Robert S. Babayi (Reg. No. 33,471), Gary L. Schaeffer (Reg. No. 34,502), Andrew C. Aitken (Reg. No. 36,729), Julie A. Petruzzelli (Reg. No. 40,769), Michael A. Sartori (Reg. No. 41,289), W. David Wallace (Reg. No. 42,210), Jeffri A. Kaminski (Reg. No. 42,709), Cameron Tousi (Reg. No. 43,197), Charles C. P. Rories (Reg. No. 43,381), Ralph P. Albrecht, (Reg. No. 43,466), Fei-Fei Chao (Reg. No. 43,538), Jeffrey W. Gluck (Reg. No. 44,457), Keith G. Haddaway (Reg. No. 46,180), and Eric J. Weierstall (Reg. No. 46,331), all at Suite 1000, 1201 New York Avenue, N.W., Washington, D.C. 20005-3917, Telephone: (202) 962-4800, Telefax: (202) 962-8300.

Address all correspondence to VENABLE, P.O. Box 34385, Washington, D.C. 20043-9998.

The undersigned hereby authorizes the U.S. attorneys named herein to accept and follow instructions from the undersigned's assignee, if any, and/or, if the undersigned is not a resident of the United States, the undersigned's domestic attorney, patent attorney or patent agent, as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and the undersigned. In the event of a change in the person(s) from whom instructions may be taken, the U.S. attorneys named herein will be so notified by the undersigned.

DECLARATION FOR UNITED STATES PATENT APPLICATION
POWER OF ATTORNEY, DESIGNATION OF CORRESPONDENCE ADDRESS

Attorney Docket
31671-169944 RK

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature: Yoshimi Homma Date: March 5, 2001.
First Joint Inventor: Yoshimi HOMMA
Citizenship: Japan
Residence and Post Office Address: 6-6-26, Horai-cho, Fukushima-shi, Fukushima 960-8157 JAPAN JPX

Signature: Noritaka Oyama Date: March 5, 2001.
Second Joint Inventor: Noritaka OYAMA
Citizenship: Japan
Residence and Post Office Address: 21-1-102, Aza-Okubo, Watari, Fukushima-shi, Fukushima 960-8141 JAPAN JPX

Signature: Koichiro Sato Date: March 6, 2001.
Third Joint Inventor: Koichiro SATO
Citizenship: Japan
Residence and Post Office Address: 6-10-11-201, Noda-machi, Fukushima-shi, Fukushima 960-8055 JAPAN JPX